COMPOSITIONS OF ALLOSTERIC HEMOGLOBIN MODIFIERS
AND METHODS OF MAKING THE SAME

FIELD OF THE INVENTION

[0001]   The present invention relates to compositions of allosteric hemoglobin modifier compounds having low levels of impurities. The invention also relates to novel methods of preparing such compositions. Included in the present invention are improved methods for the purification of compositions of allosteric hemoglobin modifier compounds. Also included in the present invention is a method for analyzing compositions of allosteric hemoglobin modifier compounds, which enables detection and quantification of impurities.

BACKGROUND OF THE INVENTION

[0002]   Hemoglobin is a tetrameric protein which delivers oxygen via an allosteric mechanism. There are four binding sites for oxygen on the hemoglobin molecule, as each protein chain contains one heme group. Each heme group contains a substituted porphyrin and a central iron atom. The iron atom in heme can be in the ferrous (+2) or ferric (+3) state, but only the ferrous form binds oxygen. The ferrous-oxygen bond is readily reversible. The binding of the first O_2 molecule to hemoglobin enhances the binding of additional O_2 to the same hemoglobin molecule. In other words, O_2 binds cooperatively to hemoglobin. Thus, binding of the first oxygen to a heme requires much greater energy than the second oxygen molecule, binding of the third oxygen requires even less energy, and the fourth oxygen requires the lowest energy for binding. Hemoglobin A, the principal hemoglobin in adults consists of two α and two β subunits arranged with a two-fold symmetry. The α and β dimers rotate during oxygen release to open a large central water cavity. The allosteric transition that involves the movement of the alpha beta dimer takes place between the binding of the third and fourth oxygen.

[0003]   Using well-known equipment such as the AMINCO™ HEM-O-SCAN, an oxygen dissociation curve can be plotted to determine the affinity and degree of cooperativity (allosteric action) of hemoglobin. In the plot, the Y-axis represents the percent of hemoglobin oxygenation and the X-axis represents the partial pressure of oxygen in millimeters of mercury (mm Hg). If a horizontal line is drawn from the 50% oxygen saturation point and a vertical line is drawn from the intersection point of the horizontal

line with the curve to the partial pressure X-axis, a value commonly known as the P_{50} is determined. This is the partial pressure (mm Hg) at which the hemoglobin sample is 50% saturated with oxygen. Under physiological conditions (i.e. 37°C, pH 7.4, and a partial pressure of carbon dioxide of 40 mm Hg), the P_{50} value for normal adult hemoglobin is around 26.5 mm Hg. If a lower than normal P_{50} value is obtained for the hemoglobin being tested, the oxygen dissociation curve is considered to be "left-shifted" and the presence of high affinity hemoglobin is indicated. Conversely, if a higher than normal P_{50} value is obtained for the hemoglobin being tested, the oxygen dissociation curve is considered to be "right-shifted" and the presence of low affinity hemoglobin is indicated. Such low affinity hemoglobin will lose oxygen more easily at lower pressures of oxygen, and therefore may be useful to deliver oxygen to tissues more efficiently.

[0004] It has been suggested that influencing the allosteric equilibrium of hemoglobin may be a viable method to treat diseases that are influenced by oxygen delivery. For example, the conversion of hemoglobin to a high affinity state is generally regarded to be beneficial in treating problems associated with deoxyhemoglobin S (sickle cell anemia.). The conversion of hemoglobin to a low affinity state is believed to be of general utility in a variety of disease states in which tissues suffer from low oxygen tension, such as ischemia, radio-sensitization of tumors, carbon monoxide poisoning, fetal oxygen delivery and the restoration of the oxygen affinity of stored blood.

[0005] Figures 1A-1D depict the chemical structures of a variety of compounds which have a "right-shifting" allosteric effect on hemoglobin (referred to herein as "allosteric hemoglobin modifier compounds" or "allosteric effector compounds"). The family of compounds represented by the general structure illustrated in Figure 1D (referred to as "RSR compounds"), are representative of a large family of compounds having a strong allosteric effect. For example, one compound in this family, 2-[4-((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-methyl propionic acid (efaproxiral, also referred to as RSR13), which has the following structure, when X^+ is H^+:
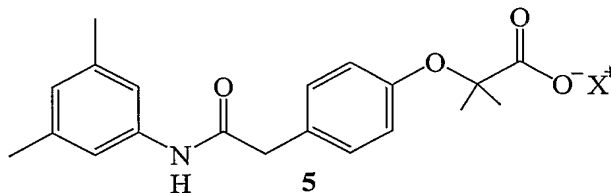

is an allosteric effector of hemoglobin, and has been shown to enhance tissue oxygenation *in vivo*. In general, efaproxiral is administered as a physiologically

acceptable salt, such as the monosodium salt; that is, X^+ is Na^+. Efaproxiral induces allosteric modification of hemoglobin, such that its binding affinity for oxygen is decreased, resulting in increased oxygen distribution to tissues by erythrocytes. Efaproxiral has been reported to enhance fractionated radiation therapy in mice bearing the Lewis lung carcinoma. See Teicher (1996) Drug Dev. Res. 38:1-11. Enhancement of the effect of radiation was observed in EMT6 mouse mammary tumors by treatment with efaproxiral plus oxygen breathing, with the absence of enhanced radiation effects in normal tissues. Rockwell and Kelley (1998) Rad. Oncol. Invest. 6:199-208. Additionally, mouse fibrosarcoma tumor growth has been shown to be reduced by the combination of efaproxiral and radiation relative to radiation alone. See Teicher (1996) Drug Dev. Res. 38:1-11; Khandelwal et al. (1996) Rad. Oncol. Invest. 4:51-59. This family of compounds, together with their utility and methods for using them are described in a number of patents including, U.S. Pat. No. 5,661,182, issued August 26, 1997, U.S. Pat. No. 5,290,803, issued March 1, 1994, U.S. Pat. No. 5,382,680, issued January 17, 1995, U.S. Pat. No. 5,432,191, issued July 11, 1995, U.S. Pat. No. 5,648,375, issued July 15, 1997, U.S. Pat. No. 5,677,330, issued October 14, 1997, U.S. Pat. No. 5,731,454, issued March 24, 1998, U.S. Pat. No. 5,122,539, issued June 16, 1992, U.S. Pat. No. 5,927,283, issued July 27, 1999, U.S. Pat. No. 5,827,888, issued October 27, 1998, U.S. Pat. No. 5,049,695, issued September 17, 1991, U.S. Pat. No. 5,591,892, issued January 7, 1997, U.S. Pat. No. 5,049,695, issued September 17, 1991, U.S. Pat. No. 5,250,701, issued October 5, 1993, U.S. Pat. No. 5,248,785, issued September 28, 1993, U.S. Pat. No. 5,705,521, issued January 6, 1998, and U.S. Pat. No. 5,525,630, issued June 11, 1996. Each of these references is specifically incorporated herein by reference in its entirety.

[0006]   As a result of the general utility and importance of these compounds a number of methods have been developed to synthesize them. Two of the principal methods developed to date are compared in Figure 2 using the synthesis of the sodium salt of 2-[4-((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-methyl propionic acid (also referred to herein as efaproxiral sodium and efaproxiral-Na) (5), for purposes of illustration. With reference to Figure 2, in the first method developed (referred to as Process A), efaproxiral-Na (5) was synthesized as the free acid (6), which was then treated with base to provide the sodium salt (5). (Randad et al. (1991) J. Med. Chem. 34:752-757). In the second method (referred to as Process B), this compound was synthesized as the ethyl ester (4), which was then saponified to provide the sodium salt

(5). (Witt *et al.*, DE 2,432,560, published January 22, 1976). Process A is highly exothermic, not easily amenable to commercial scale manufacture and uses a halogenated hydrocarbon solvent. Process B eliminates the use of a halogenated hydrocarbon solvent and is more amenable to commercial scale manufacture and thus is the preferred method. The primary drawback of Process B, however, is the unexpected generation of the polymeric impurity poly (ethyl methacrylate) and precursors to this compound, which are referred to herein collectively as PEM, which is formed in Step 2 via the following mechanism.

Ethyl 2-bromoisobutyrate

ethyl methacrylate (EM)

poly(ethylmethacrylate) or PEM
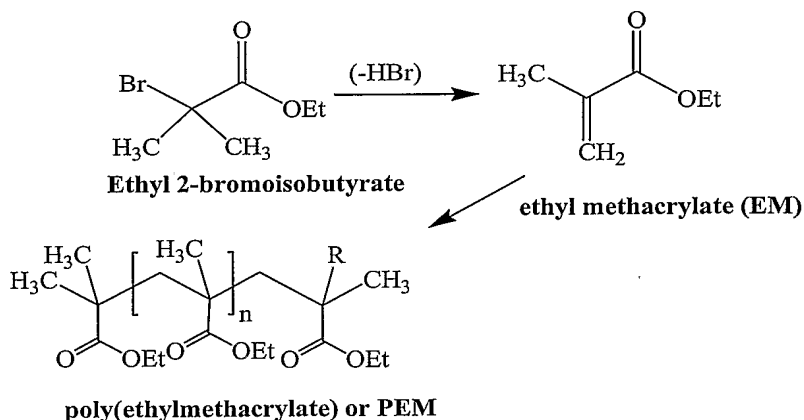
Scheme 1

[0007]   In the manufacture of the efaproxiral sodium (5) via Process B poly (ethyl methacrylate) is typically formed in concentrations of from approximately 0.5% (5000 parts per million (ppm)) to 9% (90,000 ppm) by weight.

[0008]   Despite the general utility and importance of these compounds in treating disease, problems remain in generating pharmaceutical grade compositions. Specifically, the compounds are administered to patients in a sterile intravenous (IV) solution preparation. In the process of testing these compounds, difficulty with the drug product manufacturing (IV solution formation) has been traced to the PEM byproduct generated during their synthesis as outlined above. Thus, there is a need for methods to reduce the level of polymeric impurity in preparations of allosteric hemoglobin modifier compounds in order for use in patients. There also remains a need for compositions of allosteric hemoglobin modifying compounds with lower amounts of impurities in general. The present invention provides an improved process for making highly pure allosteric effector compounds.

[0009]   Another problem associated with the prior art methods is that there is currently no effective way to measure the low levels of impurities capable of causing failure in the IV

solutions prepared from compositions, which are comprised of these compounds. As noted above, a particularly undesirable impurity is PEM. Prior art methods for measuring PEM are deficient in several respects, particularly in that they are unable to detect very low levels of PEM. Current methods for detecting and measuring PEM include ^{1}H NMR, gel permeation chromatography (GPC) or size exclusion chromatography (SEC); MALDI-TOF mass spectrometry; ultraviolet (UV) analysis; and infrared (IR) analysis. The latter two techniques are accurate for mixtures containing ≥2-4% PEM w/w in efaproxiral ethyl ester (4) or efaproxiral sodium (5). The former techniques can be used for determining the concentration of ≥0.5% w/w PEM. For example, to analyze intermediates with ≥0.5% w/w PEM, ^{1}H NMR can be used by comparing the integration of the PEM methylene proton signal to the ethoxy-methylene proton signal of the efaproxiral ethyl ester (4). By multiplying the appropriate molecular weights to the respective signals of the PEM and efaproxiral ethyl ester one can develop a formula for determining the percent weight/weight (% w/w) of PEM. Muguruma *et al.* describe a method for the quantitative analysis of poly(methylmethacrylate) (PMMA) in drug substances using pyrolysis-gas chromatography (PY/GC). Using this method, Muguruma were able to detect levels of PMMA ≥ 0.1 wt% with a precision of approximately 4.5% at a level of 0.1%. (Mugurma *et al.* (July 1999) LC-GC International, pp.432-436).
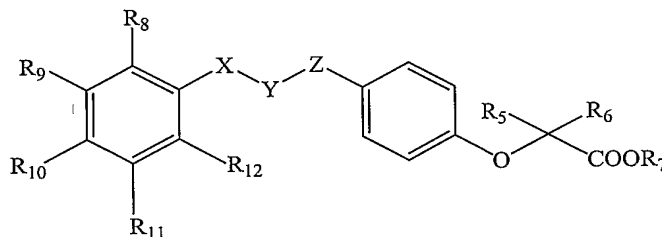
[0010]   For analysis of highly pure compositions of allosteric hemoglobin modifiers however, none of the prior art techniques can be used because the limit of detection is not low enough. The improved processes of the instant invention produce compositions of allosteric hemoglobin modifiers having very low levels of impurities (≤100 ppm (0.0100% w/w) of PEM in efaproxiral-Na). Consequently, there remains a need for a method for analyzing these compounds which has a low detection limit and good specificity to measure very low levels of PEM, as well as other polymeric impurities with adequate sensitivity. Since pyrolysis/gas chromatography/mass spectrometry (PY/GC/MS) has been used for identification of polymers in relatively intractable matrices, it was evaluated to determine whether it would be useful for trace level analysis of polymers in efaproxiral-Na. Extensive development led to the discovery of a method for quantitation of trace levels of PEM (limit of quantitation = 10 ppm). The PY/GC/MS method described herein is a novel analytical technique that utilizes single ion monitoring and an isotopically labeled PEM internal standard to provide the sensitivity, precision, accuracy and reproducibility required for the detection and quantitation of a trace level impurity. This technique can be extended to the analysis of compositions of allosteric

5

hemoglobin modifiers containing polymeric impurities other than PEM in the event that the method of synthesis illustrated in Figure 3 is modified.

[0011]   It is therefore an object of this invention to provide compositions of allosteric hemoglobin modifying compounds having lower amounts of polymeric impurities, particularly PEM, as well as, lower amounts of impurities in general.

[0012]   It is also an objective of the present invention to provide improved methods for the synthesis of compositions of allosteric hemoglobin modifying compounds having lower amounts of polymeric impurities.

[0013]   It is another object of the present invention to provide improved methods for purification of compositions of allosteric hemoglobin modifying compounds prepared by any known synthetic method, in particular by the method disclosed herein.

[0014]   Finally, it is an objective of the present invention to provide a method for analyzing compositions of allosteric hemoglobin modifying compounds, which enables detection and quantification of low levels of impurities, particularly polymeric impurities.

SUMMARY OF THE INVENTION

[0015]   The present invention includes novel compositions of allosteric hemoglobin modifier compounds that are substantially free of impurities, particularly polymeric impurities. The compositions of allosteric hemoglobin modifier compounds included within the scope of this invention are generally represented by the following formula:
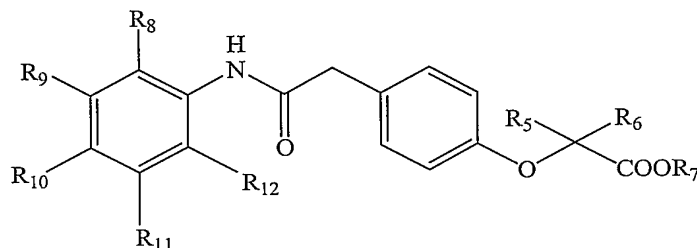

wherein

X and Z are independently selected from the group consisting of CH_2, CO, NH or O, and Y is selected from the group consisting of CO or NH, with the caveat that X, Y, and Z must all be different from each other;

R_5 and R_6 are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-12} alkyl groups, carboxylic acid and ester groups, substituted or unsubstituted aromatic or heteroaromatic groups, or alkyl moieties of part of an aliphatic ring connecting R_5 and R_6;

R_7 is a selected from the group consisting of hydrogen, a cationic counterion, selected from the group including, but not limited to sodium, potassium or ammonium, a metal, or a substituted or unsubstituted C_{1-6} alkyl group; and

R_{8-12} are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-3} alkyl groups, or alkyl moieties of an aromatic or aliphatic ring incorporating two of the R_{8-12} sites.

[0016] In a preferred embodiment of the invention, the allosteric hemoglobin modifying compounds are generally represented by the following formula:


wherein

R_5 and R_6 are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-12} alkyl groups, carboxylic acid and ester groups, substituted or unsubstituted aromatic or heteroaromatic groups or alkyl moieties of part of an aliphatic ring connecting R_5 and R_6;

R_7 is a selected from the group consisting of hydrogen, a cationic counterion, including but not limited to sodium, potassium or ammonium, a metal, or a substituted or unsubstituted C_{1-6} alkyl group; and

R_{8-12} are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-3} alkyl groups, or alkyl moieties of an aromatic or aliphatic ring incorporating two of the R_{8-12} sites.

[0017] More preferably R_5 and R_6 are independently selected from H or CH_3 and R_7 is selected from hydrogen or a cationic counterion as defined above.

[0018] In the most preferred embodiment of the invention the allosteric hemoglobin modifier compound is 2-[4-((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-methyl propionic acid (efaproxiral) (5).

7
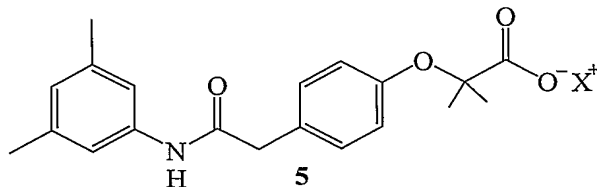
[0019]   An impurity includes any substance that does not belong in the allosteric hemoglobin modifier composition. Typically, the impurities present in the compositions are a result of the process employed to produce the allosteric hemoglobin modifier compound. For example, polymeric impurities are produced by the polymerization of one of the starting materials used to synthesize these compounds via the method exemplified by Process B. In a preferred embodiment of the invention the allosteric hemoglobin modifier composition is the sodium salt of efaproxiral (5) containing less than 0.010% non-polymeric impurities and less than 100 ppm (0.01%) of polymeric impurities, and more specifically the polymeric impurity PEM. In a most preferred embodiment, the allosteric hemoglobin modifier composition contains less than 80 ppm (0.008%) of polymeric impurities, specifically the polymeric impurity PEM.

[0020]   The present invention includes methods for the preparation of compositions of allosteric hemoglobin modifier compounds that are substantially free of polymeric impurities. The improved method for preparing allosteric hemoglobin modifier compounds is shown in Figure 3, which illustrates the synthesis of efaproxiral sodium (5) as an exemplary compound. In the most preferred embodiment of the invention, the allosteric hemoglobin modifier compounds are synthesized in reaction vessels that do not contain metals that promote the formation of polymeric byproducts and the crude synthetic products produced are then purified by extraction with methyl isobutyl ketone (MIBK) followed by an ethanol/acetone recrystallization. In this embodiment of the invention, the method for preparing compositions of allosteric hemoglobin modifier compounds is comprised of the steps of a) coupling a substituted aniline with 4-hydroxyphenylacetic acid to yield the corresponding substituted phenol; b) adding the product of step (a) to an alkyl ester halide to yield a substituted ester; and c) saponifying the substituted ester to provide the salt of the acid. As noted above, in a preferred embodiment all steps are performed in a reaction vessel that does not contain metals (referred to herein as "catalytic metals") that promote the formation of polymeric byproducts. Examples of catalytic metals that promote the formation of polymeric byproducts include, but are not limited to copper, iron, nickel, palladium and rhodium. Acceptable materials for reaction vessels include, but are not limited to glass lined stainless steel, passivated stainless steel, Hastelloy® or similar alloys low in available catalytic metals.

8

[0021]   The present invention also includes improved methods for purifying crude synthetic compositions of allosteric hemoglobin modifier compositions containing impurities, particularly polymeric impurities. The improved methods for purifying these compositions include, but are not limited to extracting the crude compositions obtained following step (c) with any water immiscible or partially immiscible solvent in which the ester formed in step (c) is soluble, including, but not limited to methyl isobutyl ketone (MIBK), isopropyl acetate, ethyl acetate, methyl ethyl ketone, chlorinated solvents selected from the group including, but not limited to chloroform and methylene chloride and recrystallizing the crude compositions with solvents including, but not limited to ethanol, acetone and mixtures of acetone/ethanol, acetone/methanol and ethanol/methanol/acetone/water. In a preferred embodiment, the recyrstallized product is further purified by filtering through a polymeric filter, wherein said polymeric filter is selected from the group including, but not limited to poly(vinylidene difluoride) (PVDF) or a cellulose ester filter.

[0022]   Finally the present invention includes a method for analyzing compositions of allosteric hemoglobin modifying compounds for impurities, particularly polymeric impurities and more particularly PEM. In this embodiment of the invention, the method for analyzing compositions of allosteric hemoglobin modifier compounds is comprised of the steps of a) pyrolyzing (PY) a composition comprised of an allosteric hemoglobin modifier compound and b) analyzing said pyrolyzed composition by gas chromatography/mass spectrometry (GC/MS). In order to improve the sensitivity of the method by the two orders of magnitude needed to measure trace amounts of polymeric impurity an isotopic internal standard is added to the sample prior to analysis. It is believed that this is the first report of the use of an internal standard, particularly an isotopic internal standard prior to analysis by PY/GC/MS for trace level analysis of polymers in drugs. The internal standard is added in an amount so as to produce a concentration approximately the same as that expected for the polymeric impurity in the compound being analyzed, which in the instant application is endogenous PEM. As noted above, the use of an internal standard, particularly an isotopic internal standard greatly improves the sensitivity while maintaining precision and accuracy in the analytical method making quantitative measurements in the 10-100 ppm range possible. Isotopes of any atom can be used including, but not limited to isotopes of hydrogen, carbon, oxygen and nitrogen. Examples of such isotopes include, but are not limited to deuterium (D), carbon 13 (^{13}C), oxygen 18 (^{18}O) and nitrogen (^{15}N). The method

described herein can be extended to the identification and quantification of any compound amenable to analysis by PY/GC/MS.

[0023]   Additional advantages and novel features of this invention shall be set forth in part in the description and examples that follow, and in part will become apparent to those skilled in the art upon examination of the following or may be learned by the practice of the invention. The advantages of the invention may be realized and attained by means of the instrumentation and in combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024]   Figures 1A-1E depict the chemical structures of a variety of compounds that have a "right-shifting" allosteric effect on hemoglobin. The family of compounds illustrated by Figure 1D (referred to as RSR compounds) are representative of a large family of compounds having a strong allosteric effect.

[0025]   Figure 2 illustrates two of the principal prior art methods developed for the synthesis of the allosteric hemoglobin modifier 2-[4-((((3,5-dimethylphenyl)amino)carbonyl)methyl) phenoxy]-2-methyl propionic acid (also known as efaproxiral).

[0026]   Figure 3 depicts the improved method of the invention developed for the synthesis of the allosteric hemoglobin modifier 2-[4-((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-methyl propionic acid (also known as efaproxiral).

[0027]   Figure 4 depicts a total ion chromatograph (TIC) for a sample of efaproxiral, showing all of the ions resulting from pyrolysis and ionization of the drug. Additionally, the sample contains low levels of PEM (less than 50 ppm) and PEM-d5 (internal standard).

[0028]   Figure 5 depicts an expansion of the TIC of Figure 4 in the region of 4.7 to 5.7 minutes showing the retention time of ions resulting from chromatographically separated PEM-d_5 (main ion: m/e 104; 5.25 minutes) and PEM (main ion: m/e 99; 5.38 minutes).

[0029]   Figures 6A and 6B depict the chromatogram (Figure 6A) and the mass spectra single ion monitoring (SIM) (Figure 6B) of PEM-d_5 using SIM mode analysis.

[0030]   Figures 7A and 7B depict the chromatogram (Figure 7A) and the mass spectra (SIM) (Figure 7B) of PEM using SIM mode analysis.

[0031]   Figure 8 depicts a standard calibration curve for the PY/GC/MS method described in Example 4.
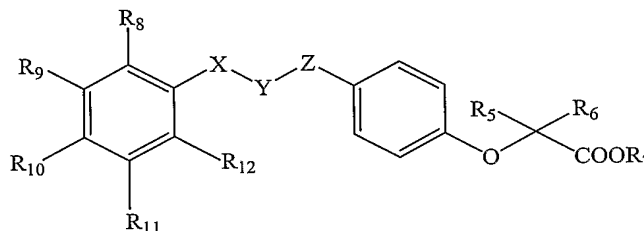

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0032]   The present invention includes novel compositions of allosteric hemoglobin modifier compounds that are substantially free of impurities, particularly polymeric impurities. In one embodiment, the allosteric hemoglobin modifier composition is 2-[4-((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-methyl propionic acid (also known as efaproxiral) containing less than 100 ppm of the polymeric impurity poly (ethyl methacrylate) (PEM). The present invention includes methods for the preparation of compositions of allosteric hemoglobin modifier compounds having very low levels of impurities. In one embodiment, the method of the invention is comprised of synthesizing the allosteric hemoglobin modifier compound according to the method described herein using a reaction vessel that does not contain available metals that promote the formation of polymeric byproducts, referred to herein as "catalytic metals."

[0033]   The present invention also includes improved methods for purifying crude synthetic compositions of allosteric hemoglobin modifier compositions containing impurities, particularly polymeric impurities. The improved methods for purifying these compositions include, but are not limited to extracting the crude compositions with any water immiscible or partially immiscible solvent in which the polymeric impurity is soluble, including but not limited to methyl isobutyl ketone (MIBK), isopropyl acetate, ethyl acetate, methyl ethyl ketone, and chlorinated solvents selected from the group including, but not limited to chloroform and methylene chloride and recrystallizing the crude compositions with solvents selected from the group including, but not limited to ethanol, acetone and mixtures of acetone/ethanol, acetone/methanol and ethanol/methanol/acetone/water. In a preferred embodiment, a solution of the recyrstallized product is further purified by filtering through a polymeric filter, wherein said polymeric filter is selected from the group including, but not limited to poly(vinylidene difluoride) (PVDF) or a cellulose ester filter.

[0034]   Finally, the present invention includes a method for analyzing compositions of allosteric hemoglobin modifying compounds for impurities, particularly PEM. In this embodiment of the invention, the method for analyzing compositions of allosteric

hemoglobin modifier compounds is comprised of the steps of a) pyrolyzing a composition comprised of an allosteric hemoglobin modifier compound and b) analyzing said pyrolyzed composition by gas chromatography/mass spectrometry (GC/MS) employing an internal standard, particularly an isotopically labeled version of the polymeric analyte as the internal standard or an analog of the polymeric analyte as the internal standard.

[0035]   Various terms are used herein to refer to aspects of the present invention. To aid in the clarification of the description of the components of this invention, the following definitions are provided.

[0036]   It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, an allosteric hemoglobin modifying compound refers to one or more allosteric hemoglobin modifying compounds. As such, the terms "a" or "an," "one or more" and "at least one," are used interchangeably herein.

[0037]   As used herein the term "**allosteric hemoglobin modifier compounds**" or "**allosteric effector compounds**" refers to a specific class of compounds, which can be generally represented by the following formula:
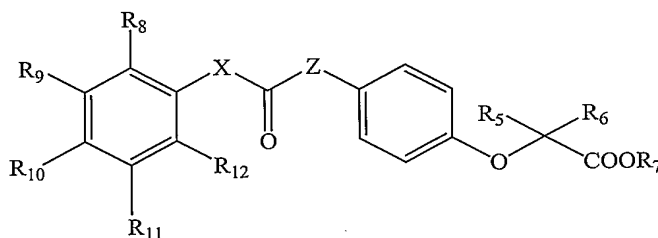

wherein

X and Z are independently selected from the group consisting of CH_2, CO, NH or O, and Y is selected from the group consisting of CO or NH, with the caveat that X, Y, and Z must all be different from each other;

R_5 and R_6 are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-12} alkyl groups, carboxylic acid and ester groups, substituted or unsubstituted aromatic or heteroaromatic groups, or alkyl moieties of part of an aliphatic ring connecting R_5 and R_6;

R_7 is a selected from the group consisting of hydrogen, a cationic counterion, selected from the group including but not limited to sodium, potassium or ammonium, a metal, or a substituted or unsubstituted C_{1-6} alkyl group; and

R_{8-12} are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-3} alkyl groups, or alkyl moieties of an aromatic or aliphatic ring incorporating two of the R_{8-12} sites.

[0038]   In a preferred embodiment of the invention, the allosteric hemoglobin modifying compounds are generally represented by the following formula:
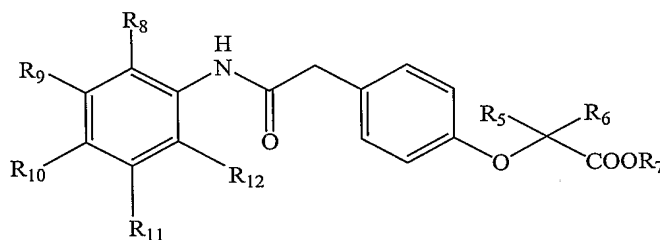

wherein

X and Z are independently selected from the group consisting of CH_2, NH or O;

R_5 and R_6 are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-12} alkyl groups, carboxylic acid and ester groups, substituted or unsubstituted aromatic or heteroaromatic groups, or alkyl moieties of part of an aliphatic ring connecting R_5 and R_6;

R_7 is a selected from the group consisting of hydrogen, a cationic counterion, including but not limited to sodium, potassium or ammonium, a metal, or a substituted or unsubstituted C_{1-6} alkyl group; and

R_{8-12} are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-3} alkyl groups, or alkyl moieties of an aromatic or aliphatic ring incorporating two of the R_{8-12} sites.

[0039]   In another preferred embodiment of the invention, the allosteric hemoglobin modifying compounds are generally represented by the following formula:
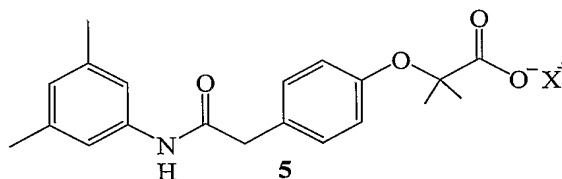

wherein

R_5 and R_6 are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-12} alkyl groups, carboxylic acid and ester groups, substituted or unsubstituted aromatic or heteroaromatic groups or alkyl moieties of part of an aliphatic ring connecting R_5 and R_6;

R_7 is a selected from the group consisting of hydrogen, a cationic counterion, including but not limited to sodium, potassium or ammonium, a metal, or a substituted or unsubstituted C_{1-6} alkyl group; and

R_{8-12} are independently selected from the group consisting of hydrogen, halogen, substituted or unsubstituted C_{1-3} alkyl groups, or alkyl moieties of an aromatic or aliphatic ring incorporating two of the R_{8-12} sites.

[0040]   More preferably R_5 and R_6 are independently selected from H or CH_3 and R_7 is selected from hydrogen or a cationic counterion as defined above.

[0041]   In the most preferred embodiment the allosteric hemoglobin modifier compound is 2-[4-((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-methyl propionic acid (efaproxiral) (5).

[0042]   As used herein the term "impurity" includes any substance that does not belong in the allosteric hemoglobin modifier composition, typically resulting from the synthesis of the allosteric hemoglobin modifier or from products of its degradation. The term impurity includes, but is not limited to polymeric impurities, such as poly (ethyl methacrylate) and precursors to this compound, which are referred to herein collectively as PEM, as well as other related impurities resulting from the synthetic process.

[0043]   As used herein the term "polymeric impurity" refers to any polymerized byproduct of the process employed to produce an allosteric hemoglobin modifier compound. In one embodiment, the polymeric impurity is selected from a compound having the following structure:
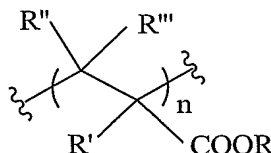
wherein

R, R', R" and R'" are independently selected from the group consisting of a substituted or unsubstituted C_{1-12} alkyl group and hydrogen; and

n is any number of units appropriate for a polymer of repeating units.

[0044]   A polymeric impurity produced as a result of the synthesis of compound (5) as described herein is poly (ethyl methacrylate) (PEM).

[0045]   Synthesis of the allosteric hemoglobin modifiers of the invention can result in polymeric byproducts of up to approximately 10% by weight depending upon reaction conditions. As noted above, in the synthesis of efaproxiral sodium (5) via Process B, the polymeric impurity PEM is typically formed in concentrations of from approximately 0.5% (5000 ppm) to 9% (90,000 ppm) by weight.

[0046]   **"Related impurities"** refer to all nonpolymeric impurities resulting from the synthetic process such as structural isomers or decomposition products. Related impurities include but are not limited to 3-monomethyl efaproxiral (3MMRS13), α-desmethyl efaproxiral (DDMRS13), monomethyl α to COOH (DMRS13), 3,4-dimethyl efaproxiral (3,4DMRS13), α ethyl efaproxiral, diacid (DA), 3,5-dimethyl aniline, amidophenol and the ethyl esters of the related impurities having the structures described in Table 1. Other related impurities would be obvious to one of skill in the art. With respect to the related impurities, the purification method described herein either meets or exceeds the acceptable levels of impurities for a high dose parenteral drug set forth in the International Conference on Harmonisation (ICH) Guidelines, which are published in the Federal Register or by the EMEA. For high dose drugs the limit for any unqualified impurity is NMT 0.05%. In a preferred embodiment of the invention, the related impurities are collectively present in an amount not exceeding about 0.75% (75000 ppm) by weight.

[0047]   By **"substantially free"** of impurities it is meant a degree of polymeric impurity not exceeding about 0.5% (5000 ppm) by weight, more preferably a degree of polymeric impurity not exceeding 0.1%, 0.07%, 0.05%, 0.025%, 0.02%, 0.015%, 0.01% (100 ppm), 0.009%, and most preferably an amount of polymeric impurity not exceeding 0.008% (80 ppm), 0.007% or 0.006% (60 ppm) by weight or less, including 0.005% (50 ppm), 0.004% (40 ppm), 0.003% (30 ppm) and 0.001% (10 ppm) and a degree of related impurities not exceeding about 0.1% by weight, more preferably a degree of related impurity not exceeding 0.09%, 0.08%, 0.07%, 0.06% or 0.05%.

[0048]   As would be known to one of skill in the art a composition which is 97% substantially free of impurities would also be considered to be 97% substantially pure, etc.

[0049]   As used herein the term **"catalyst"** refers to a substance that initiates or accelerates the rate of a chemical reaction without being consumed in the reaction. Typically, a

catalyst lowers the activation energy for a chemical reaction by providing an alternate pathway for the reaction. Catalysts that promote the formation of polymerized byproducts include, but are not limited to metals such as copper, iron, nickel, palladium and rhodium. The metals that promote the formation of polymeric byproducts are referred to herein as **"catalytic metals."**

[0050]   As used herein the term **"extraction"** or **"extracting"** refers to a liquid-liquid partition or the process of transferring a dissolved substance from one liquid phase to another (immiscible or partially miscible) liquid phase in contact with it.

[0051]   As used herein the term **"recrystallization"** or **"recrystallizing"** is a standard term which refers to a means for purifying materials by precipitation from a solvent(s).

[0052]   Note, that throughout this application various citations are provided. Each citation is specifically incorporated herein in its entirety by reference.

[0053]   The present invention includes novel compositions of allosteric hemoglobin modifier compounds that are substantially free of impurities, particularly polymeric impurities. The compositions of allosteric hemoglobin modifier compounds included within the scope of this invention are illustrated by the general structure set forth above. As noted above, an impurity includes any substance that is not the desired allosteric hemoglobin modifier composition. Typically, the impurities present in the compositions are a result of the process employed to produce the allosteric hemoglobin modifier compound. For example, the polymeric impurities are produced by the polymerization of one of the starting materials used to synthesize these compounds via the method exemplified by Process B. In a preferred embodiment of the invention the allosteric hemoglobin modifier composition is the sodium salt of efaproxiral (5) containing less than 100 ppm (0.01%) of polymeric impurities, specifically the polymeric impurity PEM and less than 1000 ppm (0.1%) of any related impurity. In a most preferred embodiment, the allosteric hemoglobin modifier composition contains less than 80 ppm of polymeric impurities and less than 500 ppm of any related impurity.

[0054]   The present invention includes methods for the preparation of compositions of allosteric hemoglobin modifier compounds that are substantially free of impurities. By "substantially free" of impurities it is meant a degree of polymeric impurity not exceeding about 0.5% (5000 ppm) by weight, more preferably a degree of polymeric impurity not exceeding 0.1%, 0.07%, 0.05%, 0.025%, 0.02%, 0.015%, 0.01% (100 ppm), 0.009%, and most preferably an amount of polymeric impurity not exceeding 0.008% (80 ppm), 0.007% or 0.006% (60 ppm) by weight or less, including 0.005% (50 ppm), 0.004% (40

ppm), 0.003% (30 ppm), and 0.001% (10 ppm) and a degree of related impurities not exceeding about 0.1% by weight, more preferably a degree of related impurity not exceeding 0.09%, 0.08%, 0.07%, 0.06% or 0.05%. In this embodiment of the invention the method for preparing compositions of allosteric hemoglobin modifier compounds is comprised of the steps of a) coupling a substituted aniline with 4-hydroxyphenylacetic acid to yield the corresponding substituted phenol; b) adding the product of step (a) to an alkyl ester halide to yield a substituted ester; and c) saponifying the substituted ester to provide the salt of the acid, wherein all steps are performed in a reaction vessel that does not contain metals that promote the formation of polymeric byproducts, referred to herein as catalytic metals. Examples of catalytic metals include, but are not limited to copper, iron, nickel, palladium and rhodium. Stainless steel (SS) is generally comprised of predominantly nickel, chromium and molybdenum. Acceptable materials for reaction vessels include, but are not limited to glass lined stainless steel, passivated stainless steel, Hastelloy® or similar alloys. The Hastelloy 276® alloy is comprised of predominantly nickel, chromium and molybdenum.

[0055]   Example 1 describes the synthesis of the sodium salt of efaproxiral (efaproxiral-Na) (5) according to the method of this invention using either a Hastelloy or SS reaction vessel. The synthesis of efaproxiral-Na (5) can be performed in any reaction vessel that does not promote the formation of the polymeric impurity PEM, including but not limited to an SS (316) reactor, a Hastelloy 276® reactor or a glass-lined SS reactor. In a preferred embodiment, the synthesis of (5) is performed in a Hastelloy 276®, SS or glass-lined SS reactor. The product prepared by the method described in Example 1 contained less than 3% by weight of the poly (ethyl methacrylate) (PEM) impurity. The use of a Monel® reaction vessel, on the other hand, resulted in a product that contained about 9% by weight PEM. Monel® is an alloy comprised predominantly of copper and nickel. Thus, the change in reaction vessel significantly reduced the amount of PEM formed during the reaction.

[0056]   The present invention also includes improved methods for purifying crude synthetic compositions of allosteric hemoglobin modifier compositions containing impurities, particularly polymeric impurities. In this embodiment of the invention, the method for preparing compositions of allosteric hemoglobin modifier compounds is comprised of the steps of a) coupling a substituted aniline with 4-hydroxyphenylacetic acid to yield the corresponding substituted phenol; b) adding the product of step (a) to an

alkyl ester halide to yield a substituted alkyl ester; c) saponifying the substituted alkyl ester to provide the salt of the acid; and d) purifying the product obtained from step c) to obtain a product which is substantially free of impurities, particularly polymeric impurities, wherein all steps are performed in a reaction vessel that does not contain metals that promote the formation of polymeric byproducts. The improved methods for purifying these compositions include, but are not limited to extracting the crude compositions with any water immiscible or partially immiscible solvent in which the polymeric impurity is soluble, including but not limited to methyl isobutyl ketone (MIBK), isopropyl acetate, ethyl acetate, methyl ethyl ketone, chlorinated solvents selected from the group including, but not limited to chloroform and methylene chloride and recrystallizing the crude compositions with solvents including, but not limited to ethanol, acetone and mixtures of acetone/ethanol, acetone/methanol and ethanol/methanol/acetone/water. In a preferred embodiment, a solution of the recyrstallized product is further purified by filtering through a polymeric filter, wherein said polymeric filter is selected from the group including, but not limited to poly(vinylidene difluoride) (PVDF) or a cellulose ester filter.

[0057]   The improved methods of this invention for purifying crude synthetic compositions of allosteric hemoglobin modifier compounds are outlined in Figure 3, using the synthesis of efaproxiral-Na (5) for purposes of illustration. Compared to prior art methods, the improved method of the invention includes addition of a purification step via extraction, a recrystallization step, a filtration step and synthesis in reaction vessels that do not contain metals that promote the formation of polymeric byproducts. Modifications were made in particular to control the levels of polymeric impurities, such as poly (ethyl methacrylate) or PEM. In one embodiment of the invention, the crude synthetic product (allosteric hemoglobin modifier compound) is extracted with a solvent such as methyl isobutyl ketone (MIBK), to remove impurities, specifically the polymeric impurity, prior to saponification. Examples of solvents that can be used to extract the crude product include any water immiscible or partially immiscible solvent in which the polymeric impurity is soluble including, but not limited to MIBK, isopropyl acetate, ethyl acetate, methyl ethyl ketone and chlorinated solvents selected from the group including, but not limited to chloroform and methylene chloride. The crude product is extracted at least one time, however in other embodiments the crude product may be extracted two or more times depending upon the impurities being removed. Exact extraction protocols can be determined without difficulty by one skilled in the art.

[0058]   Example 2 describes the purification of efaproxiral-Na (5) prepared according to the method of Example 1 by extraction with methyl isobutyl ketone (MIBK).

[0059]   Example 3 describes the purification of efaproxiral sodium (5) prepared according to the methods of Examples 1 and 2 by recrystallization with acetone/ethanol. The crude synthetic product, or the extracted crude synthetic product, is recrystallized using a solvent system such as ethanol, acetone and mixtures of acetone/ethanol, acetone/methanol and ethanol/methanol/acetone/water to reduce the amount of impurities, specifically polymeric impurities. Examples of solvent(s) that can be used to recrystallize these products include, but are not limited to ethanol, acetone and mixtures of acetone/ethanol, acetone/methanol and ethanol/methanol/acetone/water. The purified product contained less than 100 ppm of PEM.

[0060]   In the most preferred embodiment of the invention, the allosteric hemoglobin modifier compounds are synthesized in reaction vessels that do not contain metals that promote the formation of polymeric byproducts and the crude synthetic products produced are then purified by extraction with methyl isobutyl ketone (MIBK) followed by an ethanol/acetone recrystallization.

[0061]   Example 4 describes a method developed to detect and quantify trace amounts of impurities in compositions of allosteric hemoglobin compounds, specifically polymeric impurities and more specifically the polymeric impurity PEM using a GC/MS method in which the sample is pyrolyzed prior to introduction onto the GC column. (Matheson *et al.* (May 1997) American Laboratory, pp 24C-24F; Irwin (1982) in Analytical Pyrolysis, A Comprehensive Guide, Marcel Dekker, Inc.). The sample flow within the pyrolysis gas chromatography mass spectrometry (PY/GC/MS) instrument is outlined in Scheme 2 using the impurity PEM for purposes of illustration. The mass spectrometry data are collected using single ion monitoring (SIM) (Hites (1997) in Handbook of Instrumental Techniques for Analytical Chemistry, Settles, F. Ed., Prentice-Hall Inc. p. 620) to improve the signal to noise ratio, and selectively monitors a particular mass fragment arising from the ethyl methacrylate monomer. Using this method, levels of PEM as low as 10 ppm can reliably be quantified.

poly(ethylmethacrylate) or PEM

pyrolysis product

M/e = 114
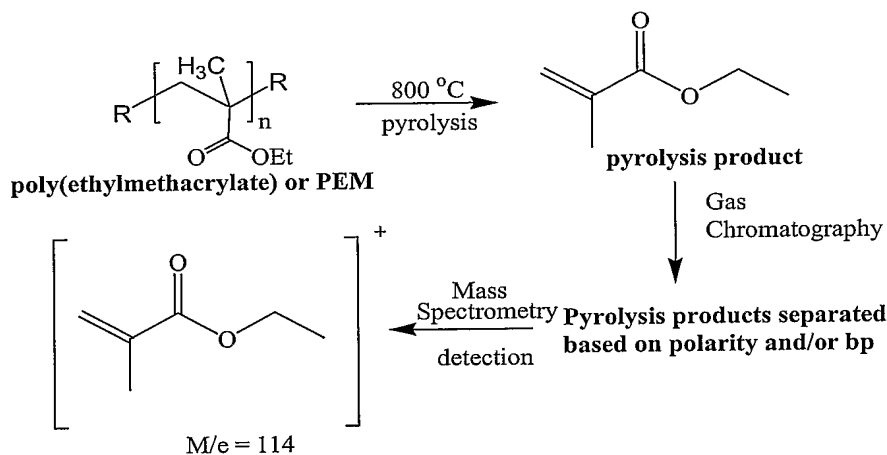
Scheme 2

[0062] An isotopic internal standard (deuterated poly (ethyl-d5 methacrylate) (PEM-d5)) is added to the sample prior to analysis. (PEM-d5 - M_w 12,555, M_n 12260, PI 1.02. It is believed that this is the first report of the use of an internal standard, particularly an isotopic internal standard prior to analysis by PY/GC/MS for a trace level analysis of polymers in drugs. Currently, no internal standard is used in the analysis of samples by this method. The internal standard is added in approximately the same expected concentration as the endogenous PEM. The use of an internal standard, particularly an isotopic internal standard greatly improves the precision and accuracy in the analytical method making quantitative measurements in the 10-100 ppm range possible. The increase in precision and accuracy is due to the fact that virtually the same compound, differing only in isotopic content and hence in molecular weight, is being subjected to the same pyrolysis conditions as the compound being analyzed. As noted above, the internal standard used in Example 4 was an isotope of hydrogen, namely PEM-d5, however isotopes of atoms other than hydrogen can be used including, but not limited to isotopes of carbon, oxygen and nitrogen. Examples of such isotopes include, but are not limited to deuterium (D), carbon 13 (^{13}C), oxygen 18 (^{18}O) and nitrogen (^{15}N). Scheme 3 depicts the monomers resulting from pyrolysis of PEM and PEM-d5 and Scheme 4 depicts the actual ions that are detected in the mass spectrometer from PEM and PEM-d5.
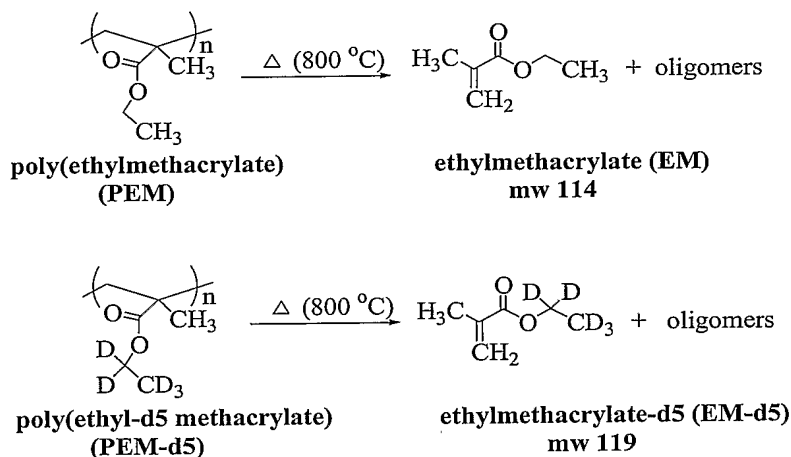
Scheme 3
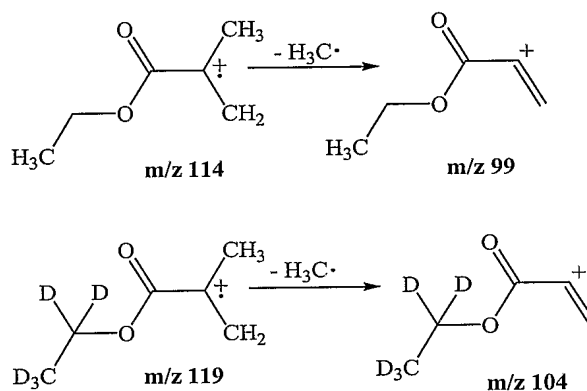
Scheme 4

[0063]   Figure 4 depicts a total ion chromatograph (TIC) for a sample of efaproxiral, showing all of the ions resulting from pyrolysis and ionization of the drug. With reference to Figure 4, it can be seen that the sample contains low levels of PEM (less than 50 ppm) and PEM-d5 (internal standard). Figure 5 depicts an expansion of the TIC of Figure 4 in the region of 4.7 to 5.7 minutes showing the retention time of ions resulting from chromatographically separated PEM-d5 (main ion: m/e 104; 5.25 minutes) and PEM (main ion: m/e 99; 5.38 minutes). Figures 6A and 6B depict the chromatogram (Figure 6A) and the mass spectra (SIM) (Figure 6B) of PEM-d5 using SIM mode analysis. Figures 7A and 7B depict the chromatogram (Figure 7A) and the mass spectra (SIM) (Figure 7B) of PEM using SIM mode analysis.

[0064]   In order to quantify the amount of ethyl methacrylate (EM) (and ultimately the amount of PEM) a standard curve is established for a range of PEM concentrations

corresponding to the acceptable range of PEM in the efaproxiral-Na sample. Each sample is then analyzed in triplicate and the concentration of PEM is determined using the linear calibration curve prepared based on the results of PY/GC/MS of the deuterated PEM. Example 5 describes the preparation of a typical standard calibration curve for the quantification of PEM by PY/GC/MS. Figure 8 depicts the standard calibration curve for the PY/GC/MS method described in Example 5.

[0065]   The invention is further illustrated by the following non-limited examples. All scientific and technical terms have the meanings as understood by one with ordinary skill in the art. The specific examples that follow illustrate the methods in which the compositions of the invention may be prepared and are not to be construed as limiting the invention in sphere or scope. The methods may be adapted to variation in order to produce compositions embraced by this invention but not specifically disclosed. Further, variations of the methods to the produce the same compositions in somewhat different fashion will be evident to one skilled in the art.

[0066]   Example 6 describes the purification of efaproxiral sodium (5) prepared according to the methods of Examples 1, 2 and 3 by filtration of the recrystallized product obtained from Example 3 through the following three filters: a polytetrafluoroethylene (PTFE) filter, a cellulose ester filter and a poly(vinylidene difluoride) (PVDF) filter. The results are set forth in Table 3. With reference to Table 3, it can be seen that PVDF filtration reduced the levels of PEM from about 55 ppm to about 9 ppm.

EXAMPLES

[0067]   Materials. The following reactions were carried out in either Hastelloy 276®, SS
(316) or glass-lined SS reactors. The Gas Chromatography/Mass Spectrometry was
performed using a Hewlett Packard 5890 or 6890 Gas Chromatograph, interfaced with a
5971, 5972 or 5973 Mass Spectrometer; equipped with cryogenic cooling option, GC/MS
ChemStation software, version A.03.00 or greater. Gas chromatography column, DB-5
Column 15 m x 0.25 mm x 0.25 μm, VWR.

Example 1.   Preparation of 2-[4-((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-
methyl propionic acid.

[0068]   Figure 3 illustrates a general five step reaction scheme for the preparation of 2-[4-
((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-methyl propionic acid which
is described in detail below.

Synthesis of Amidophenol (3)

[0069]   With reference to Figure 3, 4-hydroxyphenylacetic acid (200 kg) (2) was added to
xylene (760 L) with stirring. To this mixture, 3,5-xylidine (3,5-dimethyl aniline) (178 L)
(1) was added. The reaction mixture was heated to reflux and water was removed
azeotropically as the reaction proceeded. Upon completion, the reaction mixture was
distilled to provide amidophenol (3), which solidified upon cooling. To recrystallize,
ethanol (1180 L) and methyl isobutyl ketone (MIBK) (56 L) were added to the solid and
the mixture was refluxed until dissolution. Upon dissolution water was added (70°C, 490
L) and mixture was stirred and cooled slowly over 6 hours to about 0°C. The mixture
was then stirred for at least one hour at this temperature. The mixture was then filtered,
and the solid washed with 1:2 ethanol/water at 5°C, followed by a wash with xylene (452
L at 5°C).

Synthesis of efaproxiral Ethyl Ester (4)

[0070]   Methyl isobutyl ketone (MIBK) (827 L) was added to the crystallized
amidophenol (3) and the mixture was refluxed to azeotropically remove water. The
reaction mixture was then cooled to below 70°C, and absolute ethanol (731 L) was added,
followed by anhydrous potassium carbonate (668 kg) and ethyl 2-bromoisobutyrate (366
L). The reaction mixture was refluxed for at least 7 hours, then cooled to below 0°C.
The mixture was filtered, and the solids were washed with MIBK such that the total
volume of the wash plus the filtrate was 1208 L. The mixture was the distilled to remove

the ethanol and the volume was adjusted with MIBK to about 2163 L. The MIBK mixture was extracted with dilute aqueous base (32 kg sodium bicarbonate in 604 L of water), followed aqueous acid (63 L in 572 liters of water, and water (3 x 700 L each). The mixture was then distilled to remove MIBK and cooled to about 35°C. Heptane (about 572 L) was added and the solution was stirred while additional heptane (approximately 1145 L) was slowly added over the course of one hour. The mixture was then cooled to about 12°C, stirred for at least 2 hours and then filtered. The solid, efaproxiral ethyl ester (4) was washed with heptane (318 L).

Synthesis of efaproxiral sodium (5)

[0071]   Absolute ethanol (880 L) was first mixed with water (19 L), followed by the addition of sodium hydroxide (36 kg). This mixture was filtered, efaproxiral ethyl ester (4) was added and the reaction mixture was refluxed for at least 3 hours. Sodium hydroxide (10 N, 1 molar equivalent) was then added and reflux was maintained for at least 5 hours after the last addition. The mixture was then concentrated by distillation, and absolute ethanol (1056 L) was added. The water content was less than 0.5%. The reaction mixture was then cooled to about 40°C, then 35°C, and stirred for at least 2 hours. The mixture was then concentrated under vacuum to about 1408 L, cooled to about 10°C, and stirred for at least 5 hours. The mixture was then filtered and the solid, which consisted primarily of the sodium salt of 2-[4-((((3,5-dimethylphenyl)amino) carbonyl)methyl)phenoxy]-2-methyl propionic acid (efaproxiral sodium) (5), was washed with ethanol (282 L at 10°C).

Example 2. Purification of efaproxiral sodium (5) by extraction with MIBK

[0072]   Purified water (1658 L) was added to the product (5) (325 kg) obtained using the method described in Example 1. The mixture was distilled under vacuum at a maximum temperature of 50°C until about 423 L of solvent was removed. Another 423 L of purified water was then added and the aqueous solution was extracted with MIBK (390 L, below 30°C). The organic phase was discarded, the aqueous phase was extracted again with MIBK (228 L, below 30°C) and the organic phase was discarded.

Example 3. Purification of efaproxiral sodium (5) by recrystallization with acetone/ethanol

[0073]   The sodium salt of efaproxiral (5) synthesized as described in Examples 1 and 2 in the aqueous solution was concentrated under vacuum at a maximum temperature of 50°C to the maximum extraction of solvent, after which absolute ethanol (406 L) was added to provide a mixture having a water content of between 5 and 5.4%. The mixture was then cooled to about 47°C, acetone (975 L) was added and the mixture was stirred while maintaining the temperature. After crystallization, the mixture was stirred for at least one hour, after which an equal volume of acetone was added. The mixture was then slowly cooled to a temperature of about 15°C and stirred for at least 5 hours. The crystals were collected on a filter and washed with acetone (146 L).

Example 4. Quantitation of trace poly- ethyl methacrylate impurity in 2-[4-((((3,5-dimethylphenyl)amino) carbonyl)methyl)phenoxy]-2-methyl propionic acid

[0074]   Described below is a method for measuring trace amounts of PEM impurity in 2-[4-((((3,5-dimethylphenyl)amino) carbonyl)methyl)phenoxy]-2-methyl propionic acid using pyrolysis/gas chromatography/mass spectrometry (PY/GC/MS). PY/GC/MS is useful when the analyte in question has a large molecular weight and is either semi-volatile or nonvolatile. As discussed in detail above, typically, the sample being analyzed is heated in a controlled manner to create reproducible pyrolytically-derived compound fragments, which are then analyzed by normal GC or GC/MS. In the instant case, when a sample containing trace amounts of PEM, is subjected to pyrolysis the monomer ethyl methacrylate (EM) is generated, which is accurately measured by GC or GC/MS. An internal standard, such as deuterated PEM in methanol/methylene chloride, is introduced to provide the required precision and accuracy in the analytical test. The deuterated PEM is differentiated from the analyte PEM by its greater mass units.

[0075]   A 5 g sample of solid 2-[4-((((3,5-dimethylphenyl)amino) carbonyl)methyl)phenoxy]-2-methyl propionic acid sodium salt (5) was dissolved in methanol and a deuterated analog of PEM in methylene chloride was added to the solution as an internal standard. Additional methylene chloride was then added until a ratio of 40:60 methanol:methylene chloride was attained. The methylene chloride was required to solubilize the PEM released from the matrix. An aliquot of the sample was then placed into a prepared quartz tube packed with glass wool. After the solvent evaporated, the quartz tube was loaded into the pyrolysis unit and analyzed by

PY/GC/MS using known methods. The PEM concentration in the sample was calculated using a linear calibration curve prepared based on the results of PY/GC/MS of the deuterated PEM as described in Example 5.

Example 5. Preparation of a Standard Calibration Curve for the Quantification of PEM by PY/GC/MS

[0076]   Three standard solutions were prepared and analyzed according to the method described in Example 4. Three additional standard solutions were also prepared in order to demonstrate that acceptable linearity exceeds both ends of the calibration range specified in the method. Table 2 shows the concentration of each standard analyzed. Calibration levels corresponding to those normally used in the method are indicated by an asterisk. The standard solutions were then analyzed from the lowest to the highest concentration and a concentration calibration curve was constructed by plotting the Amount Ratio (X-axis) vs. the Response Ratio (Y-axis) as follows.

[0077]   The Amount Ratio (X-axis) is the concentration of PEM divided by the concentration of PEM-d_5 present in each standard solution. All concentrations are expressed as mass of compound sample analyzed, and are calculated by multiplying the concentrations shown in Table 2 (ng/μL) by the volume of standard solution analyzed (μL). In the instant case, the resulting units are ng in sample analyzed. Implicit in the use of the unit "ng/sample analyzed" is the assumption that the % transfer of material to the column is the same for both the internal standard and sample. The appropriateness of this assumption is demonstrated in recovery experiments.

[0078]   The "Response Ratio" (Y-axis) is calculated by dividing the measured area of the PEM peak (m/z=99) by the measured area of the PEM-d_5 peak (m/z=104). A non-weighted least-squares linear regression is performed on the paired data points (the corresponding ratios for each standard) to determine the calibration plot's line equation (slope and y-intercept). To determine sample solution concentrations ("ng on-column"), the line equation is solved for PEM concentration (i.e. y = mx + b is solved for x) as shown in Equation 1:

$$[\text{PEM}] = \frac{\left[\left(\dfrac{\text{Area PEM}}{\text{Area PEM } d_s}\right) - \left(y\text{ intercept}\right)\right]}{\text{slope}} \times \text{internal standard concentration} \quad (1)$$

wherein
    [PEM] = ng in sample analyzed concentration of PEM in sample solution
    analyzed

Area PEM = area of the m/z=99 (PEM) peak in the sample
Area PEM d_5 = area of the m/z=104 (PEM-d_5) peak in the sample
y intercept = y-intercept of the calibration curve equation
slope = slope of the calibration curve equation and
internal standard concentration = concentration of PEM-d_5 in the sample.

[0079]   To calculate concentration in the solid sample, the [PEM] result derived from

Equation 1 is multiplied by the volume of sample solution analyzed (units of μL) and

divided by the concentration of the solid sample in the sample solution (units of mg/μL).

The result is a PEM concentration in ng PEM/mg efaproxiral-Na. This number can also

be expressed as ppm.

[0080]   The calculation shown in Equation 1 is generally described in general in:

Hewlett-Packard MS ChemStation User's Guide for HP G1034C MS Chemstation

Software, Hewlett-Packard Company, Publication number G1034-90043, First Edition,

2/93. The calculation is automatically performed by the MS Chemstation software for

analyses involving internal standards. Using this procedure, a calibration curve plot for

the six standards analyzed was constructed. The resulting calibration curve which is set

forth in Figure 8, resulted in an R^2 value of 0.994.

Example 6. Purification of efaproxiral sodium (5) by filtration through a poly(vinylidene
difluoride) (PVDF) filter
    Formulation of efaproxiral-Na

[0081]   A sample of 2-[4,-((((3,5-dimethylphenyl)amino)carbonyl)methyl)phenoxy]-2-

methyl propionic acid sodium salt (efaproxiral-Na) prepared as described in Examples 1,

2 and 3 was analyzed for PEM as described in Example 5. The results from that analysis

demonstrated a PEM content of 50.3 ± 0.3 μg PEM/g efaproxiral-Na (50 ppm). This RSR

13-Na composition was then formulated for use as a drug product as follows: To a 1L

volumetric flask was added sodium chloride (2.25 g), anhydrous monobasic sodium

phosphate (135 mg) and dibasic sodium phosphate, heptahydrate (7 mg), followed by

approximately 800 mL of deionized water. The mixture was mixed until all of the solids

had dissolved. To this solution was added efaproxiral-Na (21.3 g). The mixture was

again mixed until all of the solids had dissolved. The pH of the resulting solution was

then adjusted to approximately 7.9 using 0.1N HCl. Finally, the solution was diluted to

volume using deionized water. The resulting solution represents a formulated efaproxiral

drug product.

27

Analysis of the formulated efaproxiral-Na

[0082]   To a 4 mL aliquot of the formulated efaproxiral drug product was added 20 μL of PEM-d_5 internal standard solution in a 13 x 100 mm test tube. The mixture was vortexed to homogeneity and frozen in a dry ice-isopropanol bath. The frozen sample was then lyophilized to dryness overnight. To the resulting lyophilized cake was added 400 μL of methanol followed by votexing. To this resulting mixture was added 600 μL of methylene chloride followed by vortexing. A representative sample of the prepared mixture was then centrifuged on a table-top centrifuge for five minutes. From the centrifuge tube, 5 μL of supernatant solution was transferred to a quartz tube for analysis by PY/GC/MS as described above. The determined value for PEM in the formulated drug product based on this analysis was 50.9 ± 5.0 μg PEM/g efaproxiral-Na.

Purification of formulated efaproxiral-Na by PVDF filtration

[0083]   A sample of the formulated efaproxiral drug product (50 mL) prepared as described above, was placed into a 50 mL glass syringe. To the syringe was attached one of three 0.22 μm, 25 mm disposable syringe filters (3.9 cm^2 filter area) (as set forth in Table 3). The solution was then pushed through the selected filter at a rate of approximately 8 mL/min. The entire 50 mL of filtrate was collected in a clean glass container. A 4 mL homogeneous aliquot of filtrate for each filter type was then analyzed for PEM content as described above. The results of these filtration experiments are set forth in the Table 3.

[0084]   The foregoing description is considered to be illustrative only of the principles of the invention. The words "comprise," "comprising," "include," "including," and "includes" when used in this specification and in the following claims are intended to specify the presence of one or more stated features, integers, components, or steps, but they do not preclude the presence or addition of one or more other features, integers, components, steps, or groups thereof. Furthermore, since a number of modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact composition and process shown or described above. Accordingly, all suitable modifications and equivalents may be resorted to falling within the scope of the invention as defined by the claims that follow.

Table 1. Related Impurities in RSR 13 Compositions

Impurity	Short Name	Code	Structure
A	3-monomethylanilino efaproxiral	3MMRS13	
B	α-desmethyl to COOH	DDMRS13	
C	monomethyl α to COOH	DMRS13	
D	Diacid	DA	
E	3,4-dimethyl efaproxiral	3,4DMRS13	
F	α-ethyl-efaproxiral	α-ethyl-efaproxiral	
G	3,5-dimethylaniline	3,5-DMA	
H	amidophenol	amidophenol	
I	efaproxiral ethyl ester	efaproxiral ethyl ester	

Table 2. Calibration Standard Levels

Standard Level	[PEM] (ng/μL)	[PEM-d5] (ng/μL)
1	0.985	10.4
2	1.97*	10.4
3	3.94*	10.4
4	5.91	10.4
5	7.88*	10.4
6	9.85	10.4

* Calibration levels corresponding to those typically used

Table 3. Filtration of Formulated Efaproxiral Drug Product

Filter Composition	PEM (μg/g efaproxiral-Na)
No Filter	55.6
PTFE	61.6
Cellulose Esters	15.6
PVDF	9.3